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Separation of Insulin Signaling into Distinct GLUT4 Translocation and Activation Steps

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GLUT4 (glucose transporter 4) plays a pivotal role in insulin-induced glucose uptake to maintain normal blood glucose levels. Here, we report that a cell-permeable phosphoinositide-binding peptide induced GLUT4 translocation to the plasma membrane without inhibiting IRAP (insulin-responsive aminopeptidase) endocytosis. However, unlike insulin treatment, the peptide treatment did not increase glucose uptake in 3T3-L1 adipocytes, indicating that GLUT4 translocation and activation are separate events. GLUT4 activation can occur at the plasma membrane, since insulin was able to increase glucose uptake with a shorter time lag when inactive GLUT4 was first translocated to the plasma membrane by pretreating the cells with this peptide. Inhibition of phosphatidylinositol (PI) 3-kinase activity failed to inhibit GLUT4 translocation by the peptide but did inhibit glucose uptake when insulin was added following peptide treatment. Insulin, but not the peptide, stimulated GLUT1 translocation. Surprisingly, the peptide pretreatment inhibited insulin-induced GLUT1 translocation, suggesting that the peptide treatment has both a stimulatory effect on GLUT4 translocation and an inhibitory effect on insulin-induced GLUT1 translocation. These results suggest that GLUT4 requires translocation to the plasma membrane, as well as activation at the plasma membrane, to initiate glucose uptake, and both of these steps normally require PI 3-kinase activation.

The insulin-regulated GLUT4 (glucose transporter 4), which is mainly expressed in skeletal and cardiac muscles and adipose tissues, is required for normal glucose homeostasis (10, 42, 46). In the absence of insulin, GLUT4 is stored in intracellular vesicles. In response to acute insulin stimulation, these vesicles translocate to the plasma membrane, resulting in the redistribution of GLUT4 in the plasma membrane, where GLUT4 facilitates glucose uptake (8, 45).

The signal transduction pathway initiated by insulin to translocate GLUT4 and increase glucose uptake has been extensively studied, and two signal transduction pathways have been identified in this process. One is the insulin receptor substrate (IRS)-phosphatidylinositol (PI) 3-kinase-dependent pathway, in which activated p85/p110-type PI 3-kinase phosphorylates phosphoinositides that in turn activate downstream signaling molecules, such as PDK1, Akt, and atypical protein kinase C (60). The downstream signaling molecule(s) and effectors that enable translocation of GLUT4-containing vesicles are not known. Another pathway prerequisite for GLUT4 translocation has been reported to involve the small GTP-binding protein TC10 in caveolae through tyrosine phosphorylation of cCbl (4, 12, 64). Other recent studies suggest that caveolae affect GLUT4 endocytosis rather than GLUT4 translocation to the plasma membrane (51, 55).

Reduced glucose uptake into muscle or adipose tissues in response to insulin, a condition called insulin resistance, is associated with the pathophysiology of type 2 diabetes. In skeletal muscles of type 2 diabetes patients, insulin-induced GLUT4 translocation is markedly reduced, although the impairment in upstream signaling events, such as phosphorylation of IRS-1 and Akt, is modest (68). Thus, insulin resistance in skeletal muscles may be caused mainly by an impaired signal further downstream. In adipose tissues, the expression level of IRS-1 is markedly reduced, leading to impaired activation of downstream signaling molecules, such as Akt and atypical protein kinase C, by insulin (50). The expression level of GLUT4 in adipocytes is also reduced (17). Thus, insulin resistance in adipose tissues is caused by both impaired initial-phase insulin signaling and reduced GLUT4 levels.

Numerous efforts have been made to restore insulin sensitivity in these tissues. One example of restoring glucose uptake is to overexpress GLUT4 to overcome attenuated insulin signaling and/or reduced endogenous GLUT4 expression. However, when GLUT4 was overexpressed in transgenic rodents, excess GLUT4 leaked from the intracellular storage pool and translocation machinery, resulting in the plasma membrane localization of GLUT4 and increased glucose uptake even without insulin stimulation (1, 54). When GLUT4 was overexpressed to a level that lowered the postprandial blood glucose level in transgenic insulin-resistant model rodents, they tended to develop hypoglycemia on fasting (7, 19, 20, 39, 48). Thus, the overexpression of GLUT4 to restore the impaired glucose uptake is not an ideal therapy for insulin resistance, making alternative approaches a high priority that would be facilitated by better elucidation of the mechanisms of insulin-dependent GLUT4 trafficking and activation.

It was previously reported that a rhodamine B-labeled 10-amino-acid peptide, corresponding to the phosphoinositide binding region in the N-terminal half of gelsolin, bound both the D-3- and D-4-phosphorylated phosphoinositides, but not other phospholipids, in vitro (13). This peptide, designated

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PBP10, crossed the plasma membrane when added to the medium and disorganized F-actin in NIH 3T3 fibroblasts (13). As a result, F-actin-dependent cell motility was dramatically, but reversibly, reduced in these cells following treatment with PBP10. Since GLUT4 translocation is proposed to depend on F-actin organization (6, 33, 62) and others have shown that PBP10 stimulates secretion from hepatic cells (43), we tested the effect of PBP10 treatment on GLUT4 translocation from intracellular GLUT4-containing vesicles to the plasma membrane in 3T3-L1 adipocytes.

Here, we report that PBP10 treatment caused GLUT4, but not GLUT1, translocation to the plasma membrane without increasing glucose uptake in 3T3-L1 adipocytes, demonstrating that translocation and activation of GLUT4 are separate events, both of which are necessary to increase glucose uptake. Both insulin-dependent translocation of GLUT4 to the plasma membrane and activation of GLUT4 at the plasma membrane required PI 3-kinase activity. Establishing GLUT4 activation as separate from translocation in insulin signaling will promote a more comprehensive understanding of insulin resistance and may help to identify new therapeutic targets against insulin resistance.

MATERIALS AND METHODS

Materials. The PBP10 and rhodamine B-QRL peptides were synthesized as described previously (13). Anti-GLUT4 antibody, anti-GLUT1 antibody, and anti-IRAP (insulin-responsive aminopeptidase) antibody were generous gifts from Morris Birnbaum. Anti-Akt and anti-phosphoAkt antibodies were purchased from Cell Signaling (Beverly, Mass.). Antihemagglutinin (anti-HA) antibody was purchased from Roche (Indianapolis, Ind.). Antiphosphotyrosine and anti-Cbl antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, N.Y.). Sulfo-NHS-S-S-biotin and streptavidin-horseradish peroxidase were purchased from Pierce (Rockford, Ill.). The plasmid to express HA-tagged GLUT4 was a generous gift from S. H. Cushman. Nonimmune immunoglobulin G, Alexa Fluor 488-labeled secondary antibodies, and Vybrant-DiI were purchased from Molecular Probes (Eugene, Oreg.). All other reagents from commercial sources were of analytical grade.

Cell culture. 3T3-L1 fibroblasts were maintained and induced to differentiate into adipocytes as described previously (3). Confluent cells were incubated for 48 h in Dulbecco's modified Eagle's medium containing 0.5 mM 3-isobutyl-1-methylxanthine, 4 mg of dexamethasone/ml, 12.5 mg of triglitazone/liter, and 10% fetal bovine serum. Thereafter, the cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, which was renewed every other day. The experiments were conducted 7 to 9 days after differentiation was induced, when >90% of the cells expressed the adipocyte phenotype. HA-tagged GLUT4 was expressed in 3T3-L1 adipocytes by electroporation as described previously (35). 3T3-L1 adipocytes were serum starved overnight before each experiment.

Immunostaining and microscopy. Cells were fixed for 30 min in 4% paraformaldehyde at 37°C and washed extensively with phosphate-buffered saline (PBS), followed by incubation in 50 mM ammonium chloride at room temperature (RT) for 5 min to quench excess paraformaldehyde. The cell membranes were then permeabilized and blocked in 0.2% Triton X-100 plus 1% bovine serum albumin in PBS for 30 min at RT. The cells were incubated with primary antibodies for 1 h at RT, washed extensively, and then incubated with Alexa Fluor 488-labeled secondary antibodies for 1 h at RT and washed again. For surface labeling HA-tagged GLUT4, cells were first incubated with 2 mM KCN for 10 min. Then, the cells were incubated for 1 h at RT with anti-HA antibody, washed extensively with PBS, and incubated with a fluorescently labeled secondary antibody for 1 h at RT (2). The adipocytes that were positive for the HA tag on the cell surface were counted in randomly chosen fields. In a parallel experiment, adipocytes electroporated with the HA-tagged GLUT4 plasmid were fixed, permeabilized, and stained for the HA tag to measure the transfection efficiency. Plasma membrane sheets were prepared by sonication as described previously (18). GLUT4 localized in the plasma membrane sheets was immunostained with anti-GLUT4 antibody, followed by Alexa Fluor 488-conjugated secondary antibody. The background green fluorescence was detected by staining the membrane sheets with control immunoglobulin G instead of anti-GLUT4 antibody. The membrane sheets were identified by staining them with Vybrant-DiI on a Leica DM-IRBE inverted epifluorescence microscope. The amount of GLUT4 in the membrane sheets was quantified by measuring the intensities of green fluorescence in multiple fields and by subtracting the background with Openlab software (Improvision, Lexington, Mass.). The intensity of fluorescence detected in insulin-treated cells was set to 100%. For deconvolution microscopy, images were acquired by using a DeltaVision system (Applied Precision, Issaquah, Wash.) and analyzed by SoftWoRx (Applied Precision).

Immunoprecipitation and Western blot analysis. Cells were lysed in PBS containing 1% Triton, 0.35 mg of phenylmethylsulfonyl fluoride/ml, and 100 μM sodium vanadate, after which the lysates were centrifuged for 10 min at 15,000 × *g* and 4°C to remove insoluble materials. For immunoprecipitation, the supernatants were incubated with the appropriate antibodies, after which protein G-Sepharose beads were added. The immune complexes were then collected by centrifugation, washed with PBS containing 1% Triton, boiled in Laemmli sample buffer containing 100 mM dithiothreitol, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting was performed with an ECL system (Amersham Bioscience, Piscataway, N.J.) according to the manufacturer's instructions.

IRAP endocytosis assay. IRAP endocytosis was measured by its protection from cleavage by a membrane-impermeable reagent as described previously (18). Briefly, 3T3-L1 adipocytes were incubated with or without insulin in KRPH buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM $NaPO_4$, 20 mM HEPES, pH 7.4) at 37°C for 20 min. The subsequent procedures were executed at 4°C. Insulin bound to the cell surface receptors was removed by a mild acid wash (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM $NaPO_4$, 10 mM morpholineethanesulfonic acid, pH 6.0). IRAP distributed in the plasma membrane was biotinylated by treating the cells with 0.5 mg of sulfo-NHS-S-S-biotin/ml in KRPH for 30 min. Excess sulfo-NHS-S-S-biotin was quenched by 25 mM ethanolamine in KRPH. The cells were incubated at 37°C for 5 min to allow endocytosis in the presence or absence of PBP10 and chilled again to 4°C. Biotin, linked to IRAP remaining on the plasma membrane, was selectively cleaved by incubating the cells in cleavage buffer (50 mM glutathione, 90 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 60 mM NaOH, 0.2% bovine serum albumin, pH 8.6). The cells were washed with KRPH and subjected to immunoprecipitation with anti-IRAP antibody.

Glucose transport assay. 3T3-L1 adipocytes plated in 24-well culture dishes were serum starved as indicated above, after which they were incubated in KRP buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 5 mM $NaPO_4$, pH 7.4) for an additional 1 h prior to each treatment, except where indicated in the figure legends. The assay was initiated by adding 2-deoxy-D-[^{3}H]glucose or 3-*O*-methyl-D-[^{3}H]glucose (0.5 μCi/sample; 0.1 mmol), followed by incubation at 37°C for the appropriate time, and was terminated by washing the cells once with ice-cold KRP buffer containing 0.3 mM phloretin and then twice with ice-cold KRP buffer. The cells were then solubilized in 0.1% sodium dodecyl sulfate, and the incorporated radioactivity was determined by scintillation counting. Where indicated, insulin stimulation and glucose uptake were carried out at 27°C.

RESULTS

PBP10 treatment induced GLUT4 translocation without inhibiting GLUT4 endocytosis. As reported previously, a rhodamine B-labeled 10-amino-acid peptide, designated PBP10, which is derived from the phosphoinositide binding region in the N-terminal half of gelsolin, can cross the plasma membrane and reversibly disorganize F-actin in NIH 3T3 fibroblasts (13). Since F-actin has been implicated in GLUT4 translocation (6, 33, 62), we tested the effect of PBP10 treatment on GLUT4 translocation from intracellular GLUT4-containing vesicles to the plasma membrane in 3T3-L1 adipocytes. As shown in Fig. 1A, PBP10 treatment, like insulin treatment, triggered 3T3-L1 adipocytes to display a ring-like distribution of GLUT4, suggesting that more GLUT4 molecules localized in the plasma membrane or beneath the plasma membrane in these cells. These effects of PBP10 were almost comparable to the maximal effect of insulin in recruiting GLUT4 to the plasma membrane (Fig. 1B). In contrast, a control peptide, rhodamine

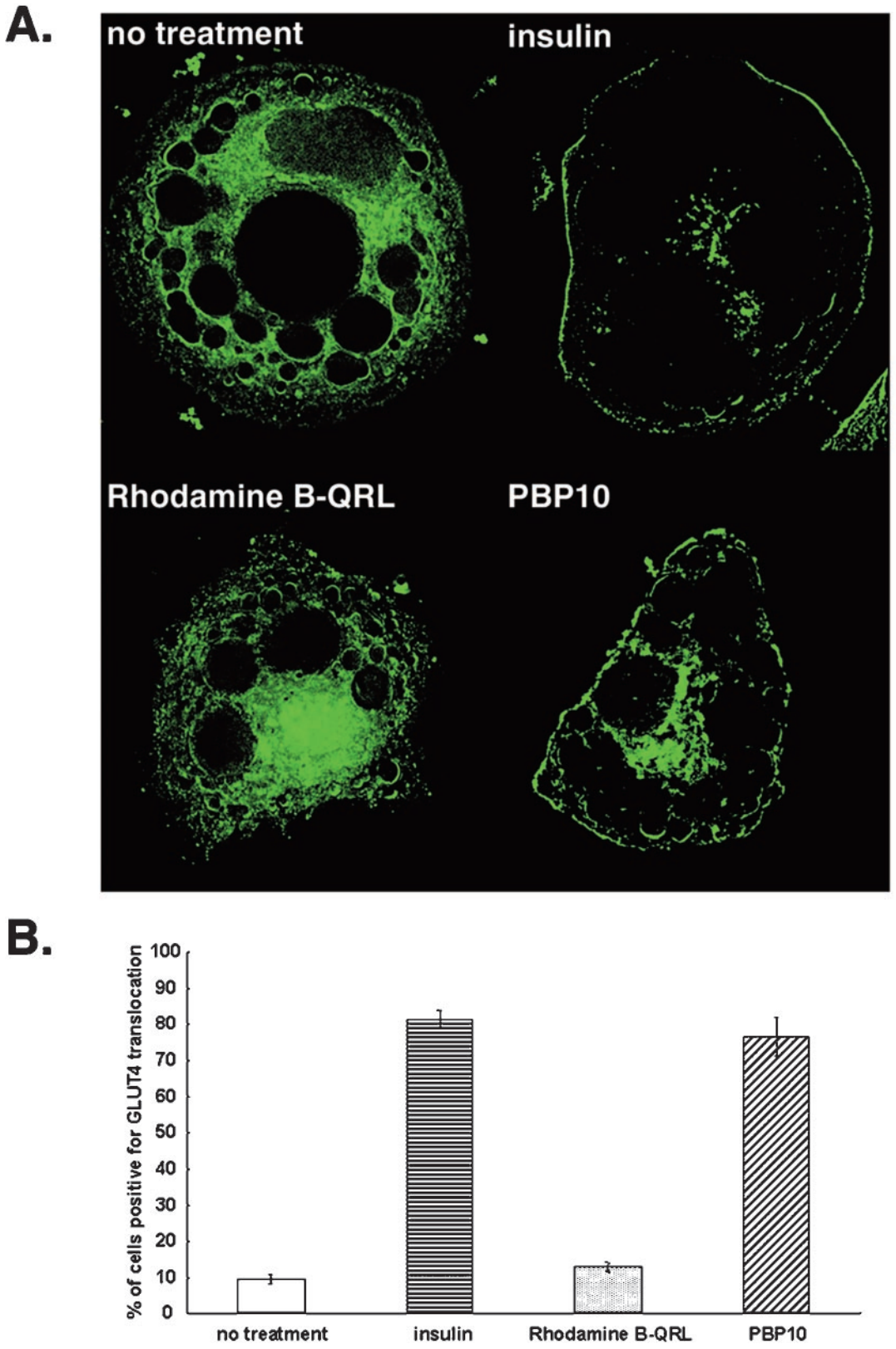

FIG. 1. PBP10 induced GLUT4 to localize in or beneath the plasma membrane in 3T3-L1 adipocytes. 3T3-L1 adipocytes were either left untreated or treated with 100 nM insulin, 40 μM rhodamine B-QRL or 40 μM PBP10 for 15 min. The cells were fixed, permeabilized, and stained for GLUT4. (A) Images were made by deconvolution microscopy in sections taken through the middle of the cells. (B) The effect of each treatment on GLUT4 translocation described in the legend to panel A was quantified by determining the number of cells exhibiting GLUT4 translocation per 100 cells observed in different fields by a deconvolution microscope. The average values ± standard errors obtained from three independent experiments are shown.

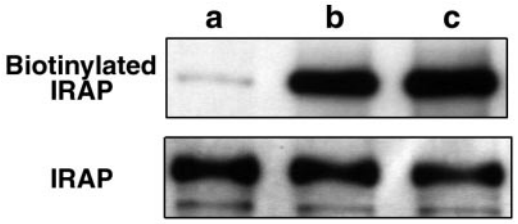

FIG. 2. PBP10 did not inhibit IRAP endocytosis. 3T3-L1 adipocytes were either left untreated (lane a) or treated with 100 nM insulin (lanes b and c). Insulin was removed, and cell surface proteins were biotinylated as described in Materials and Methods. The cells were incubated for 5 min at 37°C in the absence (lanes a and b) or presence (lane c) of PBP10 to allow endocytosis, chilled again, and incubated with a cell-impermeable biotin cleavage solution. Then, the cells were lysed, immunoprecipitated with anti-IRAP antibody, and immunoblotted either with streptavidin-horseradish peroxidase (top) or with anti-IRAP antibody (bottom). A representative result of three independent experiments is shown.

B-QRL (containing the first three residues of PBP10), which is cell permeable but fails to bind to phosphoinositides, had no effect on the subcellular distribution of GLUT4. The altered distribution of GLUT4 induced by PBP10 was observed as early as 2 min after the addition of PBP10 to the medium (data not shown).

It has been reported that GLUT4 is recycled between the intracellular vesicles and the plasma membrane at a constant rate, and insulin treatment enhances the outward movement of GLUT4 (18, 28, 38, 53, 67). Thus, the PBP10-induced increase in localization of GLUT4 in or beneath the plasma membrane could occur either by stimulating translocation or by inhibiting endocytosis. We therefore investigated whether PBP10 treatment affects endocytosis of IRAP, which resides in GLUT4-containing vesicles and is recycled between these intracellular vesicles and the plasma membrane with kinetics similar to those of GLUT4 (18, 32, 36). As reported previously (18), insulin pretreatment, followed by cell surface biotinylation and pulse-chase to allow endocytosis, significantly increased the amount of biotinylated IRAP that escaped from a cleavage of biotin moieties by a cell-impermeable agent (Fig. 2, lanes a and b). Thus, in these cells, significant amounts of IRAP were translocated to the plasma membrane by insulin stimulation and subsequently biotinylated there but then were internalized during the chase period. The presence of PBP10 during the chase period, which was long enough to exhibit its cellular effect, had no effect on the amount of IRAP internalized (Fig. 2, lanes b and c). These results suggest that the elevated amount of GLUT4 in or beneath the plasma membrane after PBP10 treatment is due to an increase in GLUT4 translocation and not to inhibition of GLUT4 endocytosis.

PBP10 treatment completes GLUT4 translocation independent of PI 3-kinase and cCbl activation. When adipocytes are stimulated with insulin, GLUT4-containing vesicles not only move to the plasma membrane but also fuse with it, resulting in the deposition of GLUT4 molecules in the plasma membrane and thereby facilitating glucose uptake (61). Since the anti-GLUT4 antibodies shown in Fig. 1 recognize the C-terminal region of GLUT4 that faces the cytoplasm both before and after translocation, it is unclear from these studies whether GLUT4 was incorporated into the plasma membrane by PBP10 treatment, present on vesicles tethered to the plasma membrane, or bound to vesicles that came close to the plasma membrane but still had no interaction with it. In order to investigate whether GLUT4-containing vesicles at least established contact with the plasma membrane after PBP10 treatment, we sonicated cells after each treatment to prepare membrane sheets, as described in Materials and Methods, and immunostained GLUT4 on the cytoplasmic surface of the remaining plasma membrane. As shown in Fig. 3A, GLUT4 was detected in the membrane sheets prepared from cells treated with either insulin or PBP10 but not from cells with no treatment or treated with rhodamine B-QRL. The delivery of GLUT4-containing vesicles to the membrane sheet was confirmed by quantifying the intensity of fluorescence for each sample (Fig. 3B). These results indicate that PBP10 treatment induced GLUT4 translocation so that GLUT4-containing vesicles were either fused with or tethered to the plasma membrane. Pretreating the cells with a relatively specific PI 3-kinase inhibitor, LY294002, before adding PBP10 did not inhibit GLUT4 translocation (Fig. 3A, image f), suggesting that, unlike insulin, PBP10-induced GLUT4 translocation occurs by a pathway independent of PI 3-kinase activity. Akt, a protein kinase activated downstream of PI 3-kinase, was not phosphorylated at any time after the addition of PBP10, suggesting that Akt activity is also not required for GLUT4 translocation by PBP10 (Fig. 4A).

Ribon and Saltiel have reported that insulin treatment causes transient tyrosine phosphorylation of cCbl, and Chiang et al. have demonstrated that activation of cCbl by tyrosine phosphorylation results in more recruitment of GLUT4 to the plasma membrane through the activation of TC10, independent of the PI 3-kinase pathway (12, 49). Therefore, we investigated the effect of PBP10 treatment on tyrosine phosphorylation of cCbl. Unlike insulin, PBP10 failed to cause tyrosine phosphorylation of cCbl (Fig. 4B), suggesting that activation of cCbl is not involved in PBP10-induced GLUT4 translocation.

In order to transport glucose, GLUT4-containing vesicles must fuse with the plasma membrane so that the extracellular domain of GLUT4 is positioned on the extracellular face of the plasma membrane. To determine if PBP10 completes proper membrane insertion of GLUT4, we expressed HA-tagged GLUT4 with the epitope tag placed in the extracellular domain of GLUT4 in 3T3-L1 adipocytes. This epitope-tagged GLUT4 has been reported to translocate identically to endogenous GLUT4 when cells are stimulated with insulin (1). We stained the cells for the HA tag without permeabilization. Since ~28% of adipocytes expressed HA-tagged GLUT4 (data not shown), only 21% of adipocytes treated with insulin exhibited the HA tag on the cell surface, which was still significantly higher than the number of cells that exhibited the HA tag on the cell surface in control or rhodamine B-QRL peptide-treated groups (Fig. 5). PBP10 treatment induced exposure of the HA tag on the cell surface at a level comparable to that observed in insulin-treated cells. These results demonstrate that GLUT4 is properly incorporated into the plasma membrane after translocation by PBP10 treatment.

PBP10 failed to increase glucose uptake without inhibiting the glucose transport potential of GLUT4. Although PBP10 did not activate signaling molecules that are utilized in the

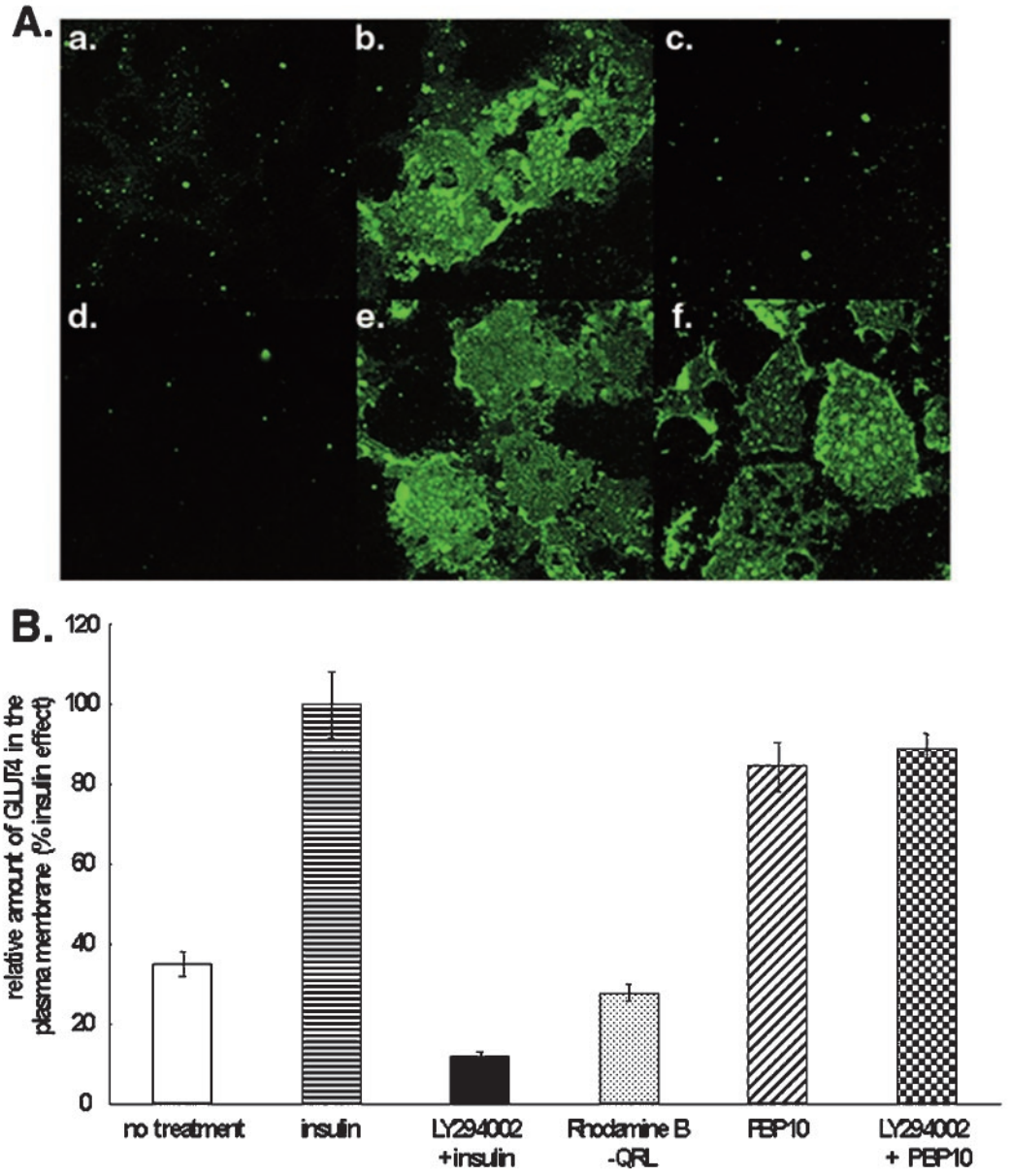

FIG. 3. PBP10 induced translocation of GLUT4-containing vesicles to the plasma membrane that was independent of PI 3-kinase activity. (A) 3T3-L1 adipocytes were pretreated either with dimethyl sulfoxide (a, b, d, and e) or 100 μM LY294002 (c and f) for 30 min. Then, the cells were left untreated (a) or incubated with either 100 nM insulin (b and c), 40 μM rhodamine B-QRL (d), or 40 μM PBP10 (e and f) for another 30 min. Membrane sheets were prepared as described in Materials and Methods. The membrane sheets were stained for GLUT4. Images obtained by an epifluorescence microscope are presented. (B) The amount of GLUT4 in the membrane sheets was quantified as described in Materials and Methods. The average values ± standard errors obtained from three independent experiments are shown.

initial phase of insulin signaling, such as PI 3-kinase, Akt, or cCbl, PBP10 was as efficient as insulin in its ability to translocate GLUT4 (Fig. 1). Thus, we investigated whether PBP10 stimulates glucose uptake in 3T3-L1 adipocytes. As shown in Fig. 6A, PBP10 treatment did not increase 2-deoxy-D-glucose uptake even at the highest concentration, where it effectively translocated GLUT4 to the plasma membrane, as shown in Fig. 1, 3, and 5. The lack of glucose uptake was not a result of damage to the membrane, since up to 40 μM PBP10 did not induce the release of lactate dehydrogenase into the medium, suggesting that the integrity of the plasma membrane remained intact (data not shown). Longer incubation with PBP10 also did not achieve glucose uptake (data not shown). PBP10 treatment also failed to increase 3-*O*-methyl-D-glucose uptake (Fig. 6B), which reflects glucose transporter function only. These results demonstrate that in the case of PBP10 treatment, GLUT4 is not able to transport glucose, even though GLUT4 has been translocated to and properly incorporated into the plasma membrane.

One possible explanation for PBP10-induced GLUT4 trans-

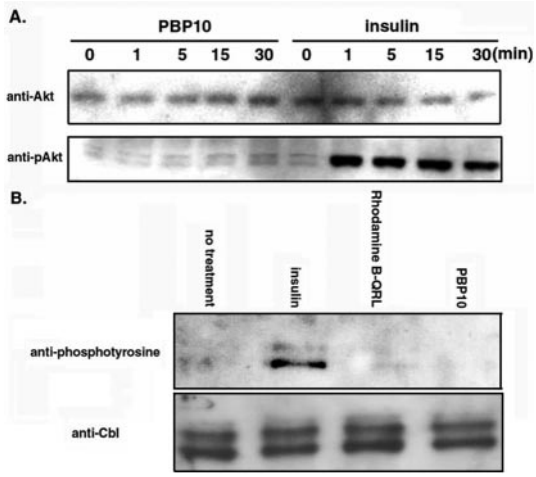

FIG. 4. PBP10 treatment activated neither Akt nor cCbl. (A) 3T3-L1 adipocytes were treated with either 100 nM insulin or 40 μM PBP10 for the indicated times. Cell lysates were immunoblotted with either anti-Akt or anti-phosphothreonine 308 Akt (anti-pAkt) antibodies. (B) 3T3-L1 adipocytes were either left untreated or treated with 100 nM insulin, 40 μM rhodamine B-QRL, or 40 μM PBP10 for 3 min at 37°C. Then, the cells were lysed and immunoprecipitated with anti-Cbl antibodies and immunoblotted with either anti-Cbl or anti-phosphotyrosine antibodies. Similar results were obtained from three independent experiments.

location without any increase in glucose uptake is that PBP10 might inhibit the transport activity of GLUT4 in the plasma membrane. To test this possibility, 3T3-L1 adipocytes were first incubated with insulin to translocate GLUT4 to the plasma membrane, and then glucose uptake was measured in the presence or absence of PBP10. As shown in Fig. 6C, after GLUT4 completed translocation following insulin stimulation, the addition of PBP10 had no effect on the insulin-induced increase in glucose uptake. These data raise the possibilities

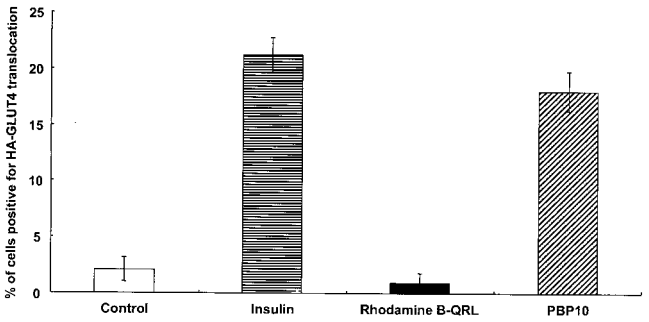

FIG. 5. GLUT4 translocated to the plasma membrane by PBP10 treatment was exposed to the extracellular surface. 3T3-L1 adipocytes were electroporated with the HA-tagged GLUT4 plasmids. Thirty hours after electroporation, a group of cells was permeabilized and stained for the HA tag to calculate the transfection efficiency, which averaged ~28% (data not shown). The remaining cells were serum starved and left untreated or incubated with either 100 nM insulin, 40 μM rhodamine B-QRL, or 40 μM PBP10 for 30 min. The cells were stained for the HA tag without permeabilization. The percentage of adipocytes that were positive for the HA tag on the cell surface per 30 to 50 adipocytes was determined in randomly chosen fields. The average values ± standard errors obtained from three independent experiments are shown.

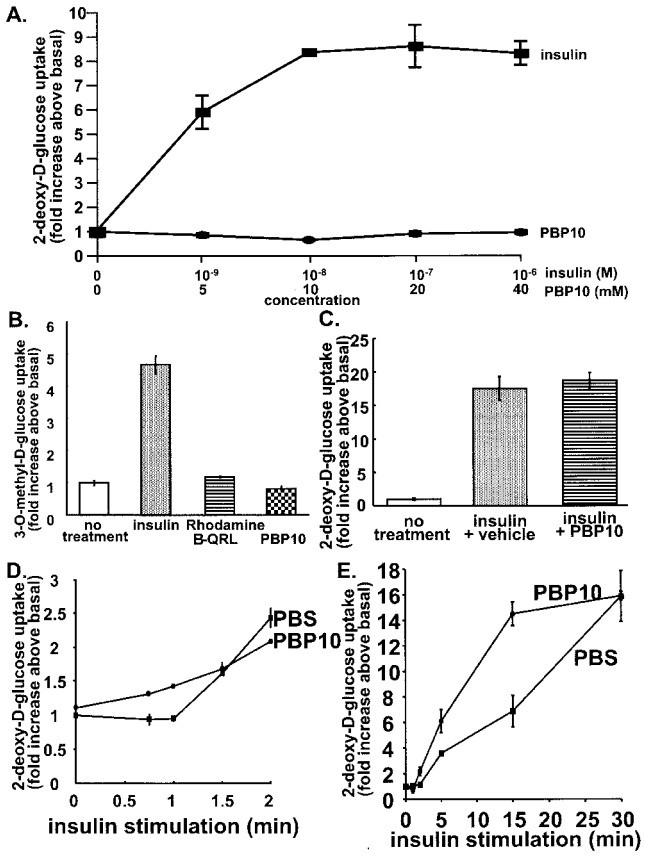

FIG. 6. Insulin treatment, but not PBP10 treatment, activated GLUT4 to facilitate glucose uptake at the plasma membrane. (A) 3T3-L1 adipocytes were treated with either insulin or PBP10 at various concentrations for 15 min. 2-Deoxy-D-glucose uptake was measured for 4 min as described in Materials and Methods. (B) 3T3-L1 adipocytes were either left untreated or treated with 100 nM insulin, 40 μM rhodamine B-QRL, or 40 μM PBP10 for 15 min. 3-*O*-Methyl-D-glucose uptake was measured for 30 s as described in Materials and Methods. (C) 3T3-L1 adipocytes were left untreated or stimulated with 100 nM insulin for 15 min. The cells were incubated for an additional 30 min with or without the addition of 40 μM PBP10, and then 2-deoxy-D-glucose uptake was measured for 4 min. (D and E) 3T3-L1 adipocytes were treated either with vehicle (PBS) or with 40 μM PBP10 for 30 min at 37°C. Then, the cells were incubated with 100 nM insulin in the presence or absence of PBP10 for various periods either at 37 (D) or at 27°C (E). 2-Deoxy-D-glucose uptake was measured for the last 1 min of insulin treatment. Similar results were obtained from three independent experiments. The error bars indicate standard errors.

that both translocation and activation of GLUT4 are required to increase glucose uptake and that PBP10 can stimulate only translocation, while insulin can stimulate both.

Activation of GLUT4 can occur separately from translocation, presumably at the plasma membrane. If glucose uptake through GLUT4 requires both GLUT4 translocation and activation and PBP10 can induce only GLUT4 translocation, then two outcomes can be expected from treating the cells with PBP10 prior to insulin, depending on the location where GLUT4 is activated by insulin stimulation. If GLUT4 is activated at the plasma membrane after translocation, then PBP10 pretreatment may decrease the time required for insulin stim-

ulation to initiate glucose uptake, since GLUT4 has already completed translocation by PBP10 when insulin is administered and insulin only needs to activate GLUT4 at the plasma membrane. On the other hand, if GLUT4 is activated by insulin before or during translocation, then PBP10 pretreatment will not be able to accelerate the time course of insulin-induced glucose uptake and GLUT4 already at the membrane would not become activated. Thus, we pretreated 3T3-L1 adipocytes either with the vehicle only or with PBP10 and measured the time course of glucose uptake after insulin was added. As shown in Fig. 6D, when insulin stimulation was executed at 37°C, the glucose uptake in PBP10-pretreated cells started to increase as early as 45 s after the addition of insulin, while the glucose uptake in vehicle-pretreated cells stayed unchanged for 1 min after the addition of insulin. Then, the amount of glucose uptake in vehicle-pretreated cells caught up with that in PBP10-pretreated cells at 1.5 min and surpassed it thereafter. However, the difference between vehicle pretreatment and PBP10 pretreatment was rather small. Elmendorf et al. have reported that insulin-induced GLUT4 translocation and glucose uptake depend strongly on the temperature (14). Therefore, we lowered the temperature of the medium to 27°C during insulin stimulation and glucose uptake so that GLUT4 translocation and activation occurred at a lower rate. As shown in Fig. 6E, PBP10-pretreated cells, which have GLUT4 already distributed in the plasma membrane at the time of insulin stimulation, showed accelerated insulin-induced glucose uptake compared to vehicle-pretreated cells. On the other hand, vehicle-pretreated cells gradually increased glucose uptake after the addition of insulin, and at 30 min, these cells exhibited an amount of glucose uptake similar to that of PBP10-pretreated cells. These results demonstrate that PBP10 treatment can bring GLUT4 to the plasma membrane in an inactive state, decreasing the necessary time for insulin to initiate glucose uptake. Based on these results, translocation and activation of GLUT4 are separate events, and GLUT4 can be activated at the plasma membrane after translocation.

Activation of GLUT4 requires PI 3-kinase activity. In order to reveal the signal transduction pathway for GLUT4 to be activated by insulin, we investigated the effects of PI 3-kinase inhibitors on insulin-induced glucose uptake after GLUT4 had completed translocation by PBP10. As shown in Fig. 7A, PBP10 pretreatment attenuated the insulin-induced increase in glucose uptake by ~40%, although insulin still increased glucose uptake significantly. When cells were treated with PBP10 first, followed by LY294002 and finally by insulin, the ability of insulin to increase glucose uptake was completely lost. Nevertheless, in these cells, PBP10 pretreatment successfully translocated GLUT4 to the plasma membrane in spite of the PI 3-kinase inhibitor, as shown by a membrane sheet assay (Fig. 7B). Wortmannin also completely inhibited insulin-induced glucose uptake in PBP10-pretreated cells (data not shown). These results strongly suggest that insulin-induced GLUT4 activation requires PI 3-kinase activity. Glucose uptake completely correlated with Akt phosphorylation (i.e., activation), which also depends on PI 3-kinase (Fig. 7A and C). Thus, PI 3-kinase activation is implicated in both translocation and activation of GLUT4 in insulin signal transduction, but a mechanism triggered by PBP10 bypasses the need for both PI 3-kinase and Akt activation to achieve GLUT4 translocation.

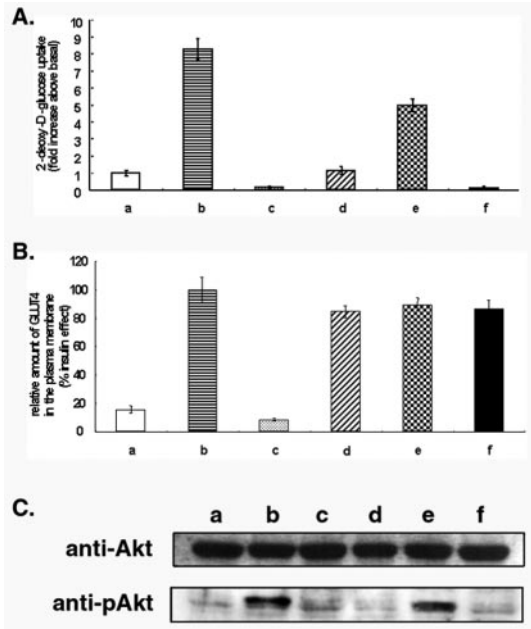

FIG. 7. Inhibition of PI 3-kinase completely abolished insulin-induced glucose uptake, although GLUT4 translocated to the plasma membrane via PBP10 pretreatment. (A) 3T3-L1 adipocytes were washed with Krebs-Ringer phosphate buffer and preincubated either with Krebs-Ringer phosphate buffer (bars a, b, and c) or 40 μM PBP10 (bars d, e, and f) for 15 min. Either dimethyl sulfoxide (DMSO) (bars a, b, d, and e) or 100 μM LY294002 (bars c and f) was added to the medium, and the mixture was incubated for 30 min. Then, the cells were either left untreated (bars a and d) or treated with 100 nM insulin (bars b, c, e, and f) for another 15 min. The cells were assayed for 2-deoxy-D-glucose uptake for 4 min. Similar results were obtained from two other independent experiments. (B and C) 3T3-L1 adipocytes were preincubated either without (bars and lanes a, b, and c) or with (bars and lanes d, e, and f) 40 μM PBP10 for 15 min. Either DMSO (bars and lanes a, b, d, and e) or 100 μM LY294002 (bars and lanes c and f) was added to the medium, and the mixture was incubated for 30 min. Then, the cells were either left untreated (bars and lanes a and d) or treated with 100 nM insulin (bars and lanes b, c, e, and f) for another 15 min. (B) Membrane sheets were prepared and immunostained for GLUT4. The amount of GLUT4 in the membrane sheets was quantified as described in Materials and Methods. The average values ± standard errors obtained from three independent experiments are shown. (C) Cell lysates were prepared and immunoblotted with either anti-Akt or anti-phosphothreonine 308 Akt (anti-pAkt) antibodies. Similar results were obtained from two other independent experiments.

PBP10 inhibits insulin-induced GLUT1 translocation. 3T3-L1 adipocytes express two distinct glucose transporters, GLUT1 and GLUT4. It has been reported that the intracellular storage sites of these glucose transporters are different. Insulin-induced GLUT1 translocation is much weaker than that of GLUT4 and involves a different signal transduction pathway (16, 31, 37, 41). In order to investigate whether PBP10 treatment primarily affects signals specific for GLUT4 translocation, we looked at the effect of PBP10 treatment on GLUT1 translocation. As shown in Fig. 8, images c, insulin treatment increased GLUT1 staining at the plasma membrane, indicating GLUT1 translocation. Neither control peptide (rhodamine B-QRL) nor PBP10 increased GLUT1 staining at the plasma membrane (Fig. 8B, image d). These results suggest that the

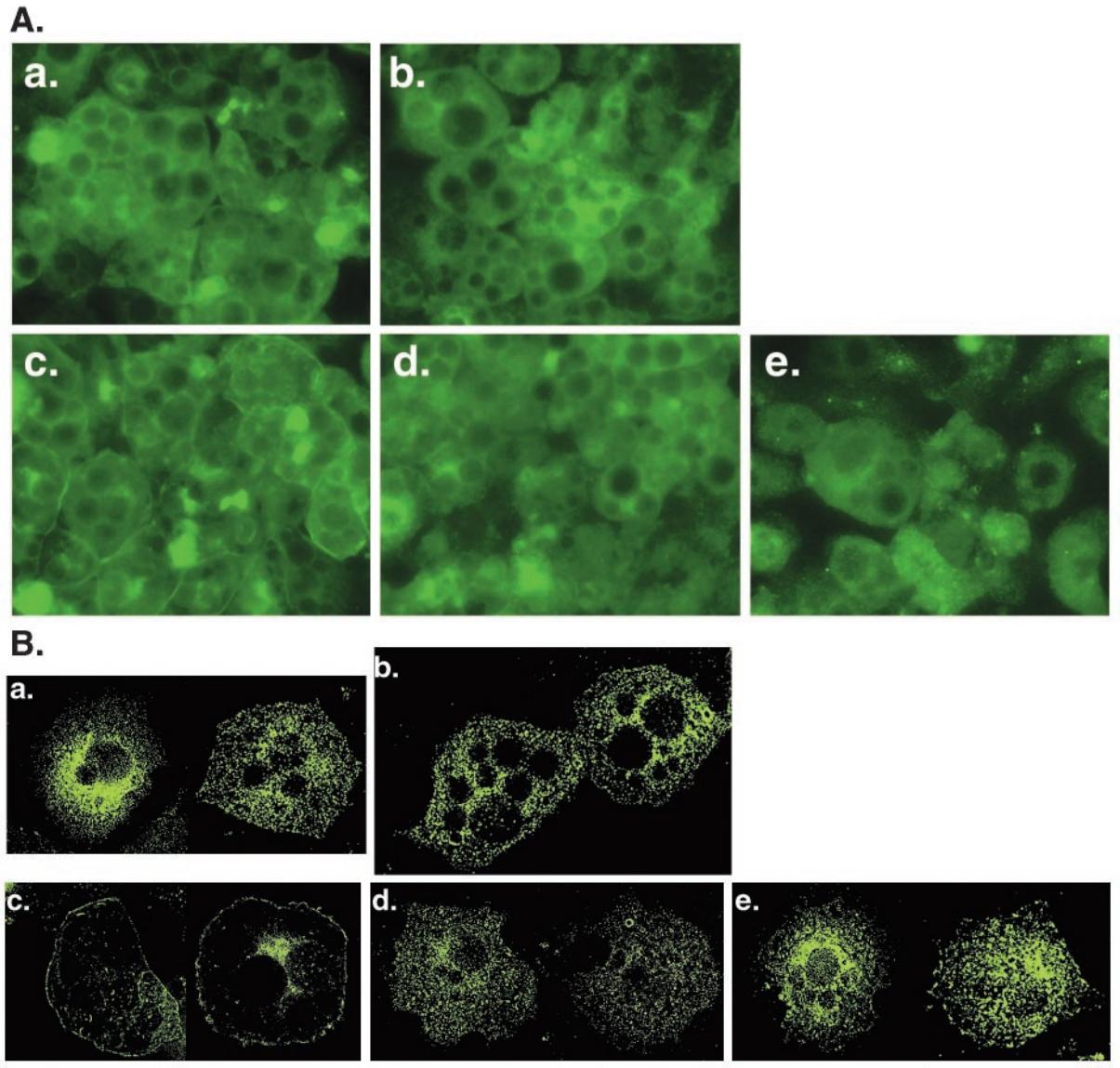

FIG. 8. PBP10 inhibited insulin-induced GLUT1 translocation. 3T3-L1 adipocytes were left untreated (images a and c) or treated with either 40 μM rhodamine B-QRL (images b) or 40 μM PBP10 (images d and e) for 15 min. Then, 100 nM insulin was administered (images c and e) for 15 min. The cells were fixed, permeabilized, and stained for GLUT1. Epifluorescence microscope images from a monolayer of cells (A) and deconvolution microscope images of individual cells (B) are exhibited. Representative results from three independent experiments are presented.

stimulatory effect of PBP10 on exocytotic events is selective for GLUT4-containing vesicles rather than translocating a broad range of intracellular vesicles nonspecifically. When cells were pretreated with PBP10 followed by insulin, insulin failed to increase GLUT1 staining at the plasma membrane (Fig. 8, images e). Thus, in the case of trafficking GLUT1-containing vesicles to the cell surface, PBP10 treatment exhibited an inhibitory effect on insulin signaling rather than mimicking insulin.

DISCUSSION

Separation of insulin signal into distinct GLUT4 translocation and activation. Although it is often presumed that insulin signaling causes the translocation of a constitutively active GLUT4 to the plasma membrane, in this report we show that GLUT4 can translocate and localize in the plasma membrane without any glucose transport activity. The inconsistency between GLUT4 translocation and glucose uptake that we report here is consistent with the results of some previous studies. Harrison et al. (22) analyzed fractions prepared by centrifugation to show that the amount of GLUT4 in the plasma membrane fraction was larger than the amount of GLUT4 labeled with [2-^{3}H]2-*N*-[4-(1-azitrifluoroethyl)benzoyl]-1,3-bis-(D-mannose-4-yloxy)-2-propylamine, a glucose analogue that can bind GLUT4 but cannot go into the cytoplasm through GLUT4, suggesting that not all GLUT4 molecules in the plasma membrane participate in glucose uptake. Holman et al. and others showed that the insulin-induced increase in the amount of

GLUT4 that was detected was smaller than the increase in glucose uptake, suggesting that the insulin-induced increase in GLUT4 in the plasma membrane cannot fully account for insulin-stimulated glucose uptake (9, 24, 30, 44, 56, 57). Sweeney et al. and Hausdorff et al. reported that inhibition of insulin signaling either by SB203580 or by a low-dose wortmannin treatment partially inhibited insulin-induced glucose uptake without any effect on GLUT4 translocation by insulin (23, 59). Nevertheless, in these reports, GLUT4 still showed some ability to transport glucose. By utilizing PBP10, we have demonstrated that GLUT4 can localize in the plasma membrane, but in an inactive form, i.e., without any glucose transport activity (Fig. 1, 3, 5, and 6A and B). This inactive GLUT4 can be activated in the plasma membrane by additional insulin stimulation, and in this case, GLUT4 starts to transport glucose sooner than GLUT4 that is newly recruited from the intracellular storage pool (Fig. 6D and E). These findings demonstrate that insulin signaling can be divided into two distinct pathways, one for GLUT4 translocation and the other for GLUT4 activation, presumably at the plasma membrane.

The molecular mechanism of PBP10-induced GLUT4 translocation. PBP10 stimulated GLUT4 translocation, instead of inhibiting GLUT4 endocytosis, to accumulate GLUT4 in the plasma membrane (Fig. 1, 2, 3, and 5). To stimulate GLUT4 translocation, PBP10 did not activate signaling molecules utilized in the initial phase of insulin stimulation, such as PI 3-kinase, Akt, or cCbl (Fig. 3 and 4). One possible mechanism is the activation of insulin-signaling molecules further downstream by PBP10 treatment. In fact, platelet-derived growth factor stimulation of 3T3-L1 adipocytes, which has been known to transiently activate the same signaling molecules as insulin stimulation downstream of IRS but fails to stimulate glucose uptake, showed significant glucose uptake after enhancement of the signal by overexpressing the platelet-derived growth factor receptor (66). Alternatively, PBP10 may stimulate another signaling pathway, similar to hyperosmotic shock or muscle contraction, to induce GLUT4 translocation (11, 65). Signaling molecules that can be utilized by both PBP10 treatment and these alternative GLUT4 translocation pathways may not be in the initial phase of the signal transduction pathway, since these alternative stimuli, but not PBP10 treatment, activate GLUT4 to increase glucose uptake. Thus, the putative target molecules that can be influenced by PBP10 treatment to cause GLUT4 translocation may be found downstream of these signal transduction pathways. One attractive candidate is the cytoskeleton. As reported previously, PBP10 treatment disorganizes cellular F actin and as a result inhibits cell motility (13). In NIH 3T3 fibroblasts, both stress fibers and cortical F actin rearrange into PBP10-positive cytoplasmic amorphous structures after PBP10 treatment. This effect of PBP10 on F-actin may affect GLUT4, since F-actin has been implicated in GLUT4 translocation (6, 33, 62). Both F-actin disruption by latrunculin or cytochalasin D and F-actin stabilization by jasplakinolide have been reported to inhibit insulin-dependent GLUT4 translocation, and the effect of TC10 on glucose uptake may be mediated by its effect on actin (29, 34). Although PBP10 treatment may disorganize F-actin, it does not depolymerize F-actin, and the amount of F-actin detected in the Triton-insoluble fractions of cell lysates did not decrease after PBP10 treatment of NIH 3T3 fibroblasts (our unpublished observations). Thus, although PBP10 treatment and F-actin-depolymerizing or F-actin-stabilizing reagents exhibit opposite effects on GLUT4 translocation in 3T3-L1 adipocytes, it is still possible to attribute PBP10-induced GLUT4 translocation to cytoskeletal changes. In addition, cross talk between microtubules and F-actin has been reported (21), and microtubules are also implicated in GLUT4 translocation (15, 26). Thus, the effect of PBP10 treatment on F-actin and/or microtubules requires further investigation to explain the mechanism of GLUT4 translocation by PBP10.

Since PBP10 was synthesized based on the sequence of the phosphoinositide-binding region in the N-terminal half of gelsolin, the cellular effect of PBP10 may be due directly to its ability to bind to phosphoinositides and not to its effect on actin. Excess PBP10 may mask phosphoinositides from other phosphoinositide-binding proteins just as the peptide sequence competes with intact gelsolin for PI 4,5-bisphosphate binding in vitro (27, 58), and also, some pleckstrin homology domains, which also bind to phosphoinositides, have been reported to inhibit phosphoinositide-dependent signaling events (47). One of the candidate phosphoinositides that PBP10 may bind is a D-3-phosphorylated phosphoinositide, such as PI 3,4,5-trisphosphate or PI 3,4-bisphosphate, produced by PI 3-kinase. However, involvement of these phosphoinositides in PBP10-dependent GLUT4 translocation is unlikely, since LY294002, a PI 3-kinase inhibitor, showed no effect on PBP10-induced GLUT4 translocation. In vitro, PBP10 is a strong activator of PI 3-kinase when PI is the substrate (25), but there is no evidence that PBP10 stimulates this activity in adipocytes, since its addition did not trigger Akt phosphorylation, which is normally a consequence of D-3-phosphorylated lipid production in these cells. Therefore, if a phosphoinositide is the target of PBP10 that enables it to translocate GLUT4, pathways involving PI 4,5-bisphosphate are more likely.

Signal transduction to activate GLUT4. Although insulin treatment induces both translocation and activation of GLUT4, PBP10 treatment stimulates only translocation. One possible explanation is that the PBP10-induced signal simply lacks the elements required for GLUT4 activation. On the other hand, it is also possible that PBP10-induced signals positively affect GLUT4 translocation but negatively affect GLUT4 activation. The latter hypothesis is suggested by the fact that PBP10 pretreatment partially, but significantly, attenuated the increase in glucose uptake by the subsequent insulin stimulation (Fig. 7A). Once GLUT4 was translocated and activated by insulin, PBP10 had no effect on insulin-stimulated glucose uptake (Fig. 6C). Since the plasma membrane, where GLUT4 is expected to become active (Fig. 6D and E), is rich in phosphoinositide, PBP10 may affect the condition of the plasma membrane by its ability to bind to (and possibly sequester) phosphoinositides and thus attenuate GLUT4 activation by insulin. Indeed, temporal interaction of GLUT4 with membrane rafts has been reported (63), and some phosphoinositide signals are reported to occur within these membrane rafts (5, 40). Thus, to hypothesize the involvement of phosphoinositide-dependent signaling in GLUT4 activation at the plasma membrane is intriguing, especially in terms of membrane rafts. Further investigation will promote understanding of the signal transduction events involved in activating GLUT4 at the plasma membrane.

Specificity in GLUT4 trafficking elucidated from PBP10 treatment. Although PBP10 treatment stimulated GLUT4 translocation, it did not induce GLUT1 translocation and even inhibited insulin-stimulated GLUT1 translocation (Fig. 8). Despite the fact that insulin treatment triggers both GLUT4 and GLUT1 (to a lesser extent) translocation in 3T3-L1 adipocytes, it has been reported that these two glucose transporters are stored in separate intracellular compartments, utilizing different machinery for translocation (31, 37, 41). In fact, overexpressing the constitutively active form of Akt induces GLUT4, but not GLUT1, translocation (16). Thus, it seems probable that, in the case of glucose transporter trafficking, PBP10 exhibited a stimulatory effect specifically on GLUT4-containing vesicles while simultaneously inhibiting the insulin signal to translocate GLUT1-containing vesicles. The inability of insulin to translocate GLUT1 in the presence of PBP10 may explain part of the attenuated glucose uptake in PBP10-pretreated cells (Fig. 6D and 7A). In fact, Rudich et al. have reported that in certain batches of 3T3-L1 adipocytes that exhibited weaker responses to insulin than ours, GLUT1 could contribute up to 50% of insulin-stimulated glucose uptake (52). The molecular mechanism of the inhibitory effect of PBP10 on GLUT1 translocation, as well as the stimulatory effect of PBP10 on GLUT4 translocation, requires further investigation.

In this report, we demonstrate activation of GLUT4, presumably at the plasma membrane, as a separate event from GLUT4 translocation downstream of insulin signaling. These findings implicate the complexity of the molecular mechanism of glucose uptake through GLUT4, which is precisely controlled in time and space in insulin signal transduction.

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